Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Bowles L, Platton S, Yartey N, et al. Lupus anticoagulant and abnormal coagulation tests in patients with Covid-19. N Engl J Med. DOI: 10.1056/NEJMc2013656

SUPPLEMENTAL APPENDIX

Lupus Anticoagulant and Abnormal Coagulation Tests in Patients with COVID-19

This appendix has been provided by the authors to give readers additional information about their work

Table of contents for supplementary materials:

List of Authors

Louise Bowles MD MRCP FRCPath*, Sean Platton MSc CSci FIBMS*, Nada Yartey BSc, MSc Minal Dave MSc, FIBMS, Kurtis Lee MSc, Daniel P Hart PhD FRCP FRCPath, Vickie MacDonald PhD MRCP FRCPath, Laura Green PhD MRCP FRCPath, Suthesh Sivapalaratnam MD PhD MRCP, K John Pasi PhD FRCP FRCPath, Peter MacCallum MD FRCP FRCPath

*Authors equally contributed in alphabetical order

Supplementary Methods

Case selection

Samples were obtained from patients admitted to the Royal London Hospital, Barts Health NHS Trust with a presumed or proven COVID-19 related presentation and investigated as part of standard of care and routine practice. Plasma was separated from consecutive citrated blood samples (3.2% sodium citrate) received in the laboratory on 2nd and 7th April 2020. Plasma was double spun according to national guidelines[1] and frozen at -80°C for 24 hours before being rapidly thawed prior to analysis, which was completed within 4 hours of sample thaw. Samples from patients who did not have a swab taken or who had a negative result for RT-PCR SARS-CoV-2 were excluded.

Control Cohort

To select a control group, historical records were examined to identify a similar number of samples to the study group with a raised APTT being investigated for LA, and in a time period before the first reported case of COVID-19 in the UK. In November and December 2019, 540 LA assays were performed in our laboratory. Of these, 43 (8%) had an APTT ≥30 seconds (local reference range 21-29 seconds). Nine of these samples were from patients who were acutely unwell, including three on critical care wards.

Coagulation assays

All assays were performed on Sysmex CS-series analyzers using standard protocols. The APTT and factor assays were performed using Siemens Actin FS, Siemens Factor Deficient Plasmas and Siemens Standard Plasma (Sysmex UK, Milton Keynes, UK). Prolonged APTT was defined as ≥30 seconds (local reference range 21-29 seconds). Factor assays were performed using a single-point assay (1/20 dilution in buffer for FVIII, 1/10 dilution in buffer for FIX, FXI and FXII).

Lupus anticoagulants assay

Lupus anticoagulant (LA) screening assays were performed using Siemens LA1 reagent for dilute Russell's Viper Venom Time (DRVVT), and Diagnostica Stago PTT-LA reagent (Diagnostica Stago, Reading, UK) for APTT. Siemens LA2 and Siemens Actin FS reagents were used as confirmatory reagents for DRVVT and APTT respectively, and mixing studies for APTT, DRVVT and LA-sensitive APTT were performed by adding equal volumes of Technoclone Platelet Poor Plasma (Pathway Diagnostics, Dorking, UK). Siemens LA1 and LA2 reagents contain a heparin neutralising agent that means that heparin levels up to 1 IU/mL have no effect on results. No other reagents used contain a heparin neutraliser.

International Society on Thrombosis and Haemostasis (ISTH) criteria were used to determine LA positivity (normalized ratio in screening test above local reference range, normalized ratio of 50/50 mix above local reference range, >10% correction in confirmatory test).[2]

Anti-Xa assay

Heparin anti-Xa assays were performed using Hyphen Biophen LRT reagents, LMWH calibrators and controls (Sysmex UK, Milton Keynes, UK).

Statistical analysis

A χ^2 test was performed to compare the frequency of lupus anticoagulants between the COVID positive and the historic cohorts with R 3.6.6 (URL https://www.R-project.org/). A

P value of ≤0.05 is considered statistically significant; all P values are two-sided. 95% confidence intervals were calculated using GraphPad Prism 8.2 (California, USA).

Supplementary Results

In the two 24-hour periods a total of 216 samples were received for coagulation screens from COVID-19 positive patients. Of these, 44 samples were found to have a prolonged APTT. Nine samples were excluded from further investigation: seven samples from patients who were already on anticoagulant therapy prior to admission; and two samples from duplicate patients. The remaining 35 samples from individual patients (16%) underwent further laboratory evaluation (see Table 1). At the time of sampling, 21 patients were tested while in critical care; 10 were from in-patients or Emergency Department patients who were admitted for more than 24 hours; 4 were from Emergency Department patients who were discharged within 24 hours.

The median age was 57 years (range 19 to 83 years), 24 were male patients. On 13th April 2020, of the 35 patients, eight had died, eight had been discharged and 19 remained in hospital of which 15 were in critical care units. One patient had a confirmed pulmonary embolus on CT pulmonary angiogram. A further patient, with end stage renal failure, had rapid deterioration in respiratory and cardiovascular function and died before a suspected pulmonary embolism could be confirmed. No postmortem was performed.

At the time of sampling, heparin was not detectable in seven patients with anti-Xa assays <0.05 IU/mL. In seven patients the anti-Xa assay was 0.05-0.19 IU/mL, in 14 it was 0.20-0.40 IU/mL and in seven it was >0.40 IU/mL (two of which were >0.50 IU/mL).

All but two patients had a platelet count ≥100 x 10^9/l. The C-reactive protein was >10 mg/L in all patients and ≥100 mg/L in 27 patients. Fibrinogen was >4.00 g/L in 34 patients. Factor VIII ranged from 99 IU/dL to 418 IU/dL with 29 samples >150 IU/dL. None of the individuals had a deficiency of factors VIII or IX. Factor XI was <50 IU/dL in five patients (37-45 IU/dL). Factor XII was ≤100 IU/dL in 34 patients and ≤50 IU/dL in 16 patients. Factor assay results shown in figure S1.

The three samples that were LA negative had factor XII levels of 55 IU/dL, 42 IU/dL and 29 IU/dL that were deemed to be the cause of the prolonged APTT in our laboratory.

In the samples that were LA positive, all samples had prolonged clotting in the 50/50 mix compared to the local reference range, and all had more than 10% correction when measured with a high phospholipid reagent compared to the low phospholipid correction, thus meeting the ISTH definition for LA positivity (see figure S1). However, for those that were positive by APTT, all samples had prolonged clotting using high phospholipid reagent (as this was how samples were identified for inclusion in the study); for those that were positive by DRVVT, 20/25 (80%) had prolonged clotting time using high phospholipid reagent.

Supplementary Discussion

Lupus anticoagulants classically prolongs the APTT as a phospholipid dependent assay and factor XII deficiency prolongs the APTT due to reduced contact activation. However, the APTT can be variably sensitive to such prolongation caused by the presence of lupus anticoagulant and factor XII, dependent upon the choice of reagents employed in the assay. The rationale for choice of reagents will depend on individual working practices of coagulation laboratories. We routinely use Actin FS which is known to be insensitive to both lupus anticoagulants[3,4] and factor XII[5,6]. Other reagent combinations that are more sensitive to LA and contact factors may result in more frequently prolonged APTT being seen in these laboratories.

A recent series of 3 cases of thrombosis in patients with COVID-19 who had anti-cardiolipin antibodies has been described[7]. However, the antibodies in these 3 patients were measured on only one occasion and were of IgG and IgA subtypes. IgA antiphospholipid antibodies are of uncertain clinical significance. In addition, other co-morbidities associated with thrombosis (cardiovascular disease, hypertension, diabetes, cancer) were present. Diagnosis of APS requires a lupus anticoagulant and/or anti-cardiolipin and/or anti-β_2-glycoprotein 1 IgG or IgM antibodies of moderate or high titer to

be present on two occasions separated by 12 weeks[8]. Further work is required before any conclusions can be drawn as to the association of SARS-CoV-2 infection with APS, if any.

In light of our study, it is possible that part of the explanation for the association between APTT prolongation and thrombosis that has been recently described in COVID-19[9] may be because of the presence of a lupus anticoagulant. It is important to note that the DRVVT reagents used contains a heparin neutraliser, neutralising any heparin effect that might otherwise lead to a false positive LA. In addition, in those patients who were LA positive, correction tests with high phospholipid, whilst fulfilling the ISTH criteria of more than 10% correction, did not correct into the reference range. This could be an indication of a very strong LA or another coagulopathy, or that there was an inherent bias in our sample selection (as they were selected for high APTT with a high phospholipid reagent) and requires further investigation.

An association between lupus anticoagulants and acquired factor XII deficiency secondary to factor XII antibodies has been previously described[10]. It is notable that the APTT prolongation in our patients was identified despite the substantial acute phase elevations of fibrinogen plus factor VIII levels, which would tend to shorten the APTT. The high fibrinogen levels and normal platelet counts and prothrombin times argues against the presence of disseminated intravascular coagulation (DIC) in these patients, and ISTH DIC scores in all patients tested were 4 or below.

The first step in the investigation of a prolonged isolated APTT is often a 50:50 mix with normal plasma, where correction suggests the presence of a factor deficiency. We observed that the APTT in these patients remained prolonged on mixing, indicating the presence of an immediate acting inhibitor (see figure S1). Although 28/35 samples contained heparin, reflecting use of routine thromboprophylaxis, when diluted by 50% the effects of heparin on the APTT should be removed, especially for samples containing low molecular weight heparin. It is a limitation of our study that we were unable to perform factor assays using multiple dilutions, which means that factor assays could be falsely

reduced by the presence of heparin and/or LA, but these would have to be at a sufficiently high level to falsely prolong the APTT in a 1/10 or 1/20 dilution using a LA-insensitive reagent. We also note that 5/35 (14%) of FXI results were below 50 IU/dL, but it is highly unlikely that these were genuine FXI deficiencies given the relative rarity of that disease, or that the levels seen (lowest value 37 IU/dL) would be associated with a bleeding diathesis.

In summary, we found that the likely explanation for the prolonged APTT in patients with COVID-19 was the presence of a lupus anticoagulant, often with associated factor XII deficiency. Further study is required to determine the role, if any, of the lupus anticoagulant (or other associated antiphospholipid antibodies) in the pathogenesis of thrombosis in this patient subgroup. Since factor XII plays no role in hemostasis and lupus anticoagulants are associated with a thrombotic rather than a bleeding tendency, this suggests that prolongation of the APTT should not be a barrier to use of anticoagulation therapies in the prevention and treatment of VTE and may be an indication for their use. Clinicians should not modify their anticoagulant approach to the prevention and treatment of thrombosis in these patients based on a prolonged APTT alone, particularly if the results of a 50/50 mix remain prolonged.

Additionally, fixed dosing of LMWH would be favoured in these circumstances over intravenous unfractionated heparin because of difficulties in monitoring the latter when the baseline APTT is prolonged, and in this setting anti-Xa levels may provide a better guide to heparin dosing.

References:

1 Mackie I, Cooper P, Lawrie A, et al. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. 2013;1–13.

2 Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. J Thromb Haemost 2009;7(10):1737–40.

3 Ratzinger F, Panic T, Haslacher H, et al. Testing lupus anticoagulants in a real-

life scenario - a retrospective cohort study. Biochem medica 2017;27(3):030705.

4 Li R, Swaelens C, Vandermijnsbrugge F, Cantinieaux B. Applying a direct aPTT ratio (PlatelinLS/ActinFS) permits to identify rapidly and reliably a bleeding-related factor deficiency or a lupus anticoagulant sequential to an isolated prolongation of aPTT in paediatric pre-operative screening. Eur J Haematol 2016;96(6):578–85.

5 Lawrie AS, Kitchen S, Purdy G, Mackie IJ, Preston FE, Machin SJ. Assessment of Actin FS and Actin FSL sensitivity to specific clotting factor deficiencies. Clin Lab Haematol 1998;20(3):179–86.

6 Bowyer A, Smith J, Woolley AM, et al. The investigation of a prolonged APTT with specific clotting factor assays is unnecessary if an APTT with Actin FS is normal. Int J Lab Hematol 2011;33(2):212–8.

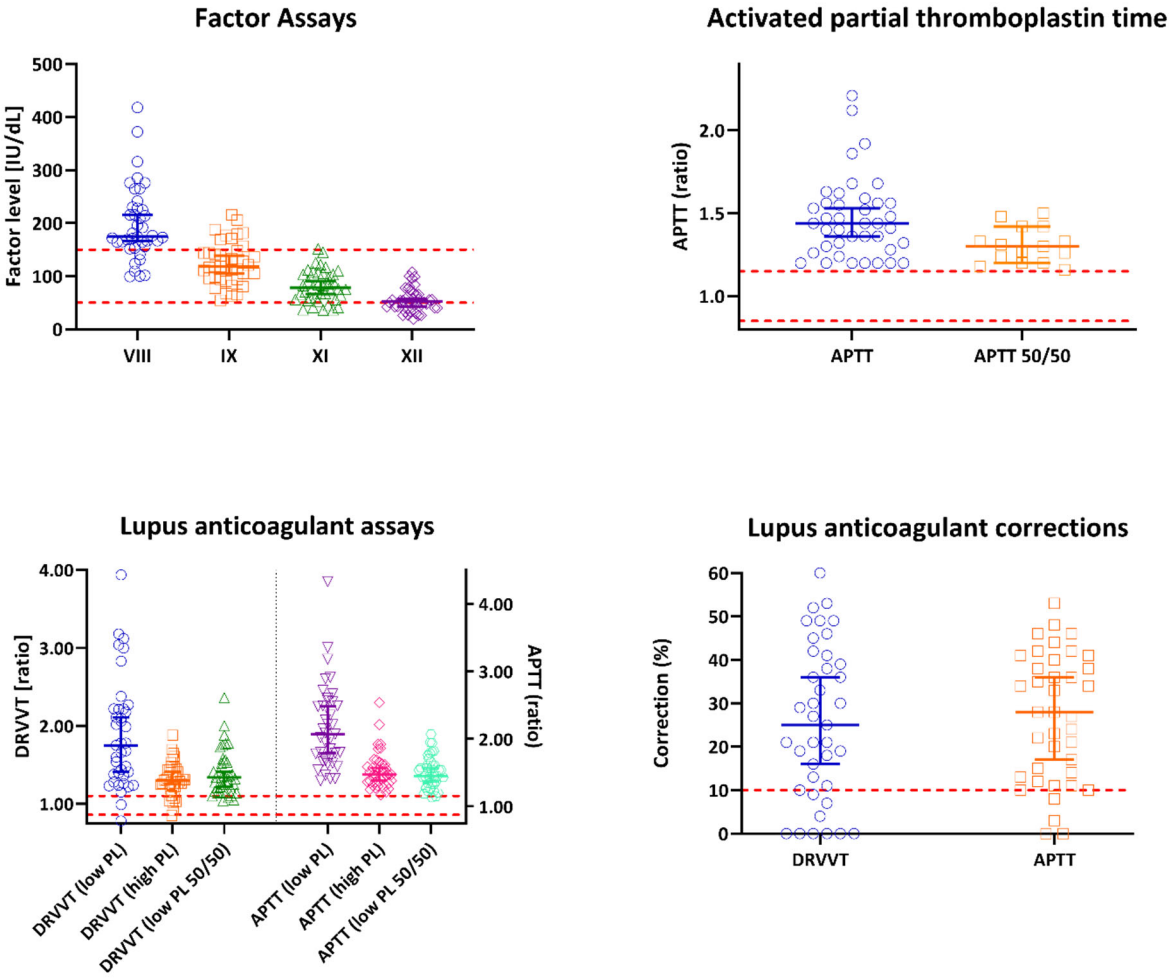
7 Yan Zhang, M.D. Meng Xiao, M.Sc. Shulan Zhang, M.D. Peng Xia, M.D. Wei Cao, M.D. Wei Jiang, M.D. Huan Chen, M.D. Xin Ding, M.D. Hua Zhao, M.D. Hongmin Zhang, M.D. Chunyao Wang, M.D. Jing Zhao, M.D. Xuefeng Sun, M.D. Ran Tian, M.D. Wei Wu, M.D. Dong Wu, M. MD. Coagulopathy and Antiphospholipid Antibodies in Patients with Covid-19. N Engl J Med 2020;1–3.

8 Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4(2):295–306.

9 Klok FA, Kruip MJHA, Meer NJM Van Der, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. Thromb Res 2020: https://doi.org/10.1016/j.thromres.2020.04.013

10 Jones DW, Gallimore MJ, Mackie IJ, Harris SL, Winter M. Reduced factor XII levels in patients with the antiphospholipid syndrome are associated with antibodies to factor XII. Br J Haematol 2000;110(3):721–6.

Figure S1:



Results of laboratory testing. Solid lines represent median and 95% confidence intervals. Dotted red lines represent local reference intervals. APTT: activated partial thromboplastin time; DRVVT: Dilute Russell's Viper Venom Time; PL: phospholipid.